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Identification of *Lactobacillus sakei* Genes Induced during Meat Fermentation and Their Role in Survival and Growth[▽]

Eric Hüfner,[1] Tobias Markieton,[1] Stéphane Chaillou,[2] Anne-Marie Crutz-Le Coq,[2] Monique Zagorec,[2] and Christian Hertel[1]*

Institute of Food Science and Biotechnology, Section Food Microbiology, University of Hohenheim, Stuttgart, Germany,[1] *and Unité Flore Lactique et Environnement Carné, Institut National de la Recherche Agronomique, Domaine de Vilvert, Jouy en Josas, France*[2]

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Lactobacillus sakei **is a lactic acid bacterium that is ubiquitous in the food environment and is one of the most important constituents of commercial meat starter cultures. In this study, in vivo expression technology (IVET) was applied to investigate gene expression of *L. sakei* 23K during meat fermentation. The IVET vector used (pEH100) contained promoterless and transcriptionally fused reporter genes mediating β-glucuronidase activity and erythromycin resistance. A genomic library of *L. sakei* 23K was established, and the clones were subjected to fermentation in a raw-sausage model. Fifteen in carne-induced fusions were identified. Several genes encoded proteins which are likely to contribute to stress-related functions. One of these genes was involved in acquisition of ammonia from amino acids, and the remaining either were part of functionally unrelated pathways or encoded hypothetical proteins. The construction and use of isogenic mutants in the sausage model suggested that four genes have an impact on the performance of *L. sakei* during raw-sausage fermentation. Inactivation of the heat shock regulator gene *ctsR* resulted in increased growth, whereas knockout of the genes *asnA2*, LSA1065, and LSA1194 resulted in attenuated performance compared to the wild-type strain. The results of our study are the first to provide an insight into the transcriptional response of *L. sakei* when growing in the meat environment. In addition, this study establishes a molecular basis which allows investigation of bacterial properties that are likely to contribute to the ecological performance of the organism and to influence the final outcome of sausage fermentation.**

Lactobacillus sakei is a ubiquitous lactic acid bacterium (LAB) and is commonly associated with the food environment. Although the organism can be isolated from various plant fermentations, e.g., sauerkraut and silage fermentation (32, 63), it is mostly isolated from the meat environment (14, 27). *L. sakei* is recognized as one of the most important components of starter cultures used for production of fermented meat products, most notably raw fermented sausages, in western Europe (28, 40). Recently, it was shown that this species is also a transient member of the human gastrointestinal microbiota (15). In addition to the fact that *L. sakei* occurs ubiquitously, it also displays notable differences in physiological and biochemical properties compared to other lactobacilli (4, 13). For example, *L. sakei* is exceptionally adaptable to changing environmental redox conditions due to its heme-dependent catalase KatA (33) and other enzymes that allow the organism to cope with deleterious oxygen by-products (13). In addition, *L. sakei* is able to proliferate at refrigeration temperatures and in the presence of high salt concentrations (up to 9% sodium chloride) (13). Tolerance for both low temperature and high salt plays a key role in meat processing in many meat manufacturing environments (14). The special status of *L. sakei* among lactobacilli is also highlighted by 16S rRNA gene sequence-based phylogenetic analysis, which shows that *L. sakei* belongs to the deepest branch within the genus *Lactobacillus* (27).

The prevalence of *L. sakei* in a variety of habitats indicates its potential to adapt to and/or to compete in different ecosystems. Recently, the 1.88-Mb genome sequence of the sausage isolate *L. sakei* 23K (14) was published (13), providing fundamental information on the genetic endowment of this organism. The genome analysis revealed potential survival strategies, as well as metabolic properties that enable *L. sakei* to effectively compete in the raw-meat environment. The existence of such unique features can be viewed as evolutionary adaptation to the meat environment (13). For example, genes involved in exogenous nucleoside salvage pathways (alternative energy source) and ABC transporters for osmo- and cryoprotective substances are present. In contrast, little is known about the regulation of gene expression of *L. sakei* in various environments. It has been argued that genes showing greater expression in a particular ecosystem ("niche-specific genes") are more likely to contribute to ecological fitness than genes expressed equally across a range of environments (53). If this is true, then only a combined knowledge of genome features and specific gene expression is required for understanding the adaptive mechanisms of *L. sakei* to the meat environment.

In vivo expression technology (IVET) has proved to be a valuable tool for the identification of genes that contribute to the performance of an organism in specific environments (for reviews see references 53 and 54). IVET permits the detection of promoters that are selectively induced in a particular habitat and has been successfully used to identify *Lactobacillus* genes that are induced during sourdough fermentation and coloni-

* Corresponding author. Mailing address: Institute of Food Science and Biotechnology, Section Food Microbiology, University of Hohenheim, Garbenstr. 28, D-70599 Stuttgart, Germany. Phone: 49 711 459 24255. Fax: 49 711 459 24199. E-mail: hertel@uni-hohenheim.de.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene
L. sakei LTH667	Raw-sausage isolate, source of *ldhL* promoter	26
L. sakei 23K	Meat isolate, plasmid-cured strain	14
L. sakei EH100	Strain 23K harboring pEH100; Em^s Cm^r GusA$^-$	This study
L. sakei EH200	Strain 23K harboring pEH200; Em^r Cm^r GusA$^+$	This study
L. sakei Blue1	*L. sakei* 23K harboring pEH100 containing an insert with in vitro promoter activity; Em^r Cm^r GusA$^+$	This study
L. sakei White1	*L. sakei* 23K harboring pEH100 containing an insert without in vitro promoter activity; Em^s Cm^r GusA$^-$	This study
L. sakei 23K(pLPV111)	*L. sakei* 23K harboring pLPV111; Em^r	This study
L. sakei RVRR3	*L. sakei rrp-3* mutant; *rrp-3*::pRV300 Em^r	49
L. sakei RVASP	*L. sakei asnA2* mutant; *asnA2*::pRV300 Em^r	This study
L. sakei RVCLPC	*L. sakei clpC* mutant; *clpC*::pRV300 Em^r	This study
L. sakei RVCTSR	*L. sakei ctsR* mutant; *ctsR*::pRV300 Em^r	This study
L. sakei RVNOD	*L. sakei* LSA1194 mutant; LSA1194::pRV300 Em^r	This study
L. sakei RVBETA	*L. sakei* LSA1065 mutant; LSA1065::pRV300 Em^r	This study
L. sakei RVTERC	*L. sakei* LSA1637 mutant; LSA1637::pRV300 Em^r	This study
L. sakei RVERFK	*L. sakei* LSA1649 mutant; LSA1649::pRV300 Em^r	This study
L. gasseri ADH	Source of the promoterless *gusA* gene	55
Plasmids		
pRV566	Derivative of the indigenous plasmid pRV500 of *L. sakei* RV332, monocopy *E. coli-Lactobacillus* shuttle vector; Em^r Ap^r; 7.29 kb	1
pRV601	Derivative of pRV566; Ap^r Em^r; 4.76 kb	This study
pRVcat	Derivative of pRV601, replacement of *bla* and *ermAM* with *cat-194*; Cm^r; 3.47 kb	This study
pFX3	Source of chloramphenicol resistance gene *cat-194*; Cm^r; 4.3 kb	70
p29TIVET	Source of the IVET cassette; Ap^r; 4.8 kb	65
p29TIVETgus	Derivative of p29TIVET, replacement of *'bglM* with *'gusA*; Ap^r; 5.3 kb	This study
pEH100	Promoter trap vector; Cm^r; 6.83 kb	This study
pEH200	pEH100 with *ldhL* promoter inserted upstream of *gusA*; Em^r Cm^r; 7.0 kb	This study
pLPV111	*E. coli-L. plantarum-L. sakei* shuttle vector; Em^r; 4.2 kb	5
pRV300	Nonreplicative delivery vector; Ap^r Em^r; 3.55 kb	39

zation of the murine gut (10, 16, 65). Recently, it has been shown using studies of mutants that several of the in vivo-induced *Lactobacillus* genes are essential for the fitness of the organism in a particular ecosystem (64). This supports the hypothesis of Rainey and Preston (53) that specific gene expression is an essential tool for bacterial adaptation.

In this paper, we describe the application of IVET to elucidate specific gene expression of *L. sakei* 23K during raw-sausage fermentation. Eight of 15 genes induced during fermentation (in carne induced [ici]) were selected for the construction of isogenic mutants. Four mutants exhibited altered growth during fermentation, indicating that the ici genes contribute to the ecological performance of *L. sakei* in raw-sausage fermentation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *L. sakei* was cultured microaerobically (2% O_2, 10% CO_2, 88% N_2) at 30°C in modified MRS (mMRS) medium containing the following (in g liter^{-1}): Bacto tryptone (Becton Dickinson), 10.0; Difco beef extract (Becton Dickinson), 8.0; Bacto yeast extract (Becton Dickinson), 4.0; glucose, 20.0; Tween 80, 1.0; $K_2HPO_4 \cdot 3H_2O$, 2.0; diammonium citrate, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.05 (pH 6.3). *Lactobacillus gasseri* was grown microaerobically at 37°C in Difco MRS medium (Becton Dickinson). *Escherichia coli* was cultured aerobically at 37°C in Luria-Bertani or SOB medium (56). When required, antibiotics were added at the following concentrations: chloramphenicol, 7 μg ml^{-1} (lactobacilli) and 20 μg ml^{-1} (*E. coli*); erythromycin, 10 μg ml^{-1} (lactobacilli) and 400 μg ml^{-1} (*E. coli*); ampicillin, 100 μg ml^{-1}. To screen clones for active promoters in vitro (active β-glucuronidase), the mMRS medium was supplemented with 100 μg ml^{-1} X-Glu (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) and 7 μg ml^{-1} chloramphenicol.

Genetic techniques. Recombinant-DNA techniques and agarose gel electrophoresis were carried out by using standard protocols (56). Plasmid DNA from *E. coli* and lactobacilli was isolated using the GenElute Plasmid Miniprep kit (Sigma-Aldrich) with the following modifications. For lactobacilli, cells of an overnight culture (5 ml) were harvested by centrifugation (9,000 × *g*, 3 min) and washed once with 1 ml of phosphate-buffered saline (PBS) (56). Cells were resuspended in 200 μl of resuspension buffer from the GenElute Plasmid Miniprep kit containing lysozyme (20 mg ml^{-1}) (Serva Electrophoresis GmbH, Germany) and mutanolysin (250 U ml^{-1}; Sigma-Aldrich) and incubated at 37°C for 1 h. Further steps were performed according to the supplier's recommendations. Genomic DNA of *L. sakei* was isolated as described previously (57). Purification of PCR products and plasmid DNA was carried out with the NucleoSpin II kit (Macherey-Nagel, Germany) according to the supplied protocol. Recombinant DNA molecules were introduced into *E. coli* and lactobacilli by electrotransformation (6, 20).

Construction of the IVET vector. All oligonucleotides used are listed in Table 2, and a scheme depicting cloning steps in the construction of IVET vector pEH100 is given in Fig. 1. The *E. coli-Lactobacillus* shuttle vector pRV601 was constructed as follows. The relevant *repA*-containing fragment was amplified from template plasmid pRV566 (1) using primers AML005 and AML006 and digested with ApaI and EcoRV. The PCR product was ligated with pRV300 cut with the same enzymes, generating pRV601 (4.76 kb). The erythromycin (*ermAM*) and ampicillin (*bla*) resistance genes of plasmid pRV601 were then removed by digestion with PagI, blunt-ended with Klenow polymerase, and then digested with KpnI. The chloramphenicol resistance gene *cat-194* from plasmid pFX3 was amplified by using primers catKpnF2 and catBspR2. The 1.1-kb PCR product was digested with KpnI and ligated with the 1.1-kb pRV601 fragment, resulting in the vector pRVcat (3.47 kb). The IVET cassette was designed on the basis of pJW100 (65), by replacing the β-glucanase gene *bglM* with the β-glucuronidase gene *gusA* of *L. gasseri* ADH (55). To do this, vector p29TIVET (65)

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)[a]	Created restriction site	Application
AML005	ATGGGCCCGTTTTGATCTCGTACAGTG	ApaI	Construction of pRV601
AML006	ATGATATCAATTCATCAGCAAGTTCTC	EcoRV	Construction of pRV601
catKpnF2	TGACTGGTACCGTTACCCTTATTATCAAGAT	KpnI	Amplification of *cat-194*
catBspR2	TGACTTTCGAATTGATCTGGAGCTGTAATAT		Amplification of *cat-194*
pJWinversFor	TAATACCTAGGCAGCTGGGCATGATGGAT	XmaJI	Amplification of p29TIVET
pJWinversRev	ACACCCTGCAGATGACTAGCTATCTATGATCAC	PstI	Amplification of p29TIVET
gusAFor	GAGAACTGCAGACTAGAAAGGAAAATCATCT	PstI	Amplification of *′gusA*
gusARev	AAAAACCTAGGTATTAATTTAATTGTTGCCA	XmaJI	Amplification of *′gusA*
IVET-EclF	TGACTGAGCTCGAGCTTCTGTTTTGGCG	SacI	Amplification of IVETgus cassette
IVET-EclR	TGACTGAGCTCCAAGATTGTTTAAGCAAAATAAG	SacI	Amplification of IVETgus cassette
ldhL1	AGTCGACGTTTGTTATTGC		Amplification of *ldhL* promoter
ldhL2	CAATTCTTCATTTCGAAAAC		Amplification of *ldhL* promoter
IVETFor	CATTCAAATATGTATCCGCTC		Sequencing primer
IVETgusRev	AGTGCCATTCATTAAAGTGT		Sequencing primer
asnFor	GATCTGCAGTAACGAATGGCTTATGAAGGAT	PstI	Creation of *L. sakei asnA2* mutant
asnRev	GATGTCGACTCATCAAATCTTCGCCTAAA	SalI	Creation of *L. sakei asnA2* mutant
asn_controlFor	ACGCTTTTTTATTTTGGAAA		Control of *L. sakei asnA2* mutant
clpCFor	GATCTGCAGTAAGGAACGCACTACGTGAACT	PstI	Creation of *L. sakei clpC* mutant
clpCRev	GATGTCGACTCAAAGTCTTGTGCACCAAC	SalI	Creation of *L. sakei clpC* mutant
clpC_controlFor	ATTTACACCAAGTGCCAAAA		Control of *L. sakei clpC* mutant
ctsRFor	GATCTGCAGTAAATTGAAATTCGACGCTCCG	PstI	Creation of *L. sakei ctsR* mutant
ctsRRev	GATGTCGACAATACCAAATTCCCCTCTTTTT	SalI	Creation of *L. sakei ctsR* mutant
ctsR_controlFor	TGCAAAGTCAGAATATCTCGG		Control of *L. sakei ctsR* mutant
terCFor	GATCTGCAGTAAGTTATTCGTTCTTCTGAAGATT	PstI	Creation of *L. sakei* LSA1637 mutant
terCRev	GATGTCGACGCTAAGACGGAATCAATCGAG	SalI	Creation of *L. sakei* LSA1637 mutant
terC_controlFor	CCTTTCTTTGAATTAAATAACTGGC		Control of *L. sakei* LSA1637 mutant
lactamFor	GATCTGCAGTAAGTTGTGGAAGTTAACGACGA	PstI	Creation of *L. sakei* LSA1065 mutant
lactamRev	GATGTCGACGCCTGCGCGATAAATCAT	SalI	Creation of *L. sakei* LSA1065 mutant
lactam_controlFor	ATGCCACTTGGTGGTGTACGC		Control of *L. sakei* LSA1065 mutant
erfKFor	GATCTGCAGTAAAAAATGAAAAAAAGCCCTTCA	PstI	Creation of *L. sakei* LSA1649 mutant
erfKRev	GATGTCGACTTAAAGAAGGCTTCACCGTG	SalI	Creation of *L. sakei* LSA1649 mutant
erfK_controlFor	GCGATTACCGCAAGCTATCC		Control of *L. sakei* LSA1649 mutant
nodFor	GATCTGCAGTAAATTAACGTCATGCGAGCCA	PstI	Creation of *L. sakei* LSA1194 mutant
nodRev	GATGTCGACCATAAGGATTTGTGAAGGCGT	SalI	Creation of *L. sakei* LSA1194 mutant
nod_controlFor	TCGGTTGAAGTATTACAAATCGT		Control of *L. sakei* LSA1194 mutant
DH05	ACGACGTTGTAAAACGACGGCCAG		Control of *L. sakei* mutants

[a] Recognition sites for restriction endonucleases are underlined.

was amplified by inverse PCR with primers pJWinversFor and pJWinversRev, containing restriction enzyme recognition sites for XmaJI and PstI, respectively. The 4.1-kb PCR product was digested with PstI and XmaJI. The gene *gusA* was amplified by using primers gusAFor and gusARev, containing recognition sites for the restriction enzymes PstI and XmaJI, respectively. The 1.8-kb PCR product containing the promoterless *gusA* and the dedicated ribosome binding site was digested with PstI and XmaJI and ligated with the 4.1-kb PCR product of p29TIVET to create plasmid p29TIVETgus. To construct the promoter trap vector pEH100, the resulting 3.4-kb IVET cassette was amplified with primers IVET-EclF and IVET-EclR, both containing the recognition site for SacI. The PCR product was digested with SacI and ligated with the dephosphorylated SacI-digested basic vector pRVcat, resulting in pEH100 (6.83 kb).

To test the functionality and stability of pEH100, the promoter of the lactate dehydrogenase gene *ldhL* (47) of *L. sakei* LTH667 (26) was amplified by using primers LDHL1 and LDHL2. The resulting PCR product was inserted into the dephosphorylated SmaI site of pEH100, generating pEH200. After transformation, the Em^r and β-glucuronidase-positive phenotype of the resulting strain, *L. sakei* EH200, was confirmed by cultivation on selective agar plates containing X-Glu. The MICs of erythromycin for *L. sakei* strains were determined by using dilution series in mMRS broth in microtiter plates, and concentrations up to 1 mg ml^{-1} were tested. Experiments to determine the stability of plasmids maintained in *L. sakei* were performed as described previously for *Lactobacillus reuteri* (29).

Construction of the genomic library. Chromosomal DNA of *L. sakei* 23K was partially digested with MseI to obtain fragments ranging from approximately 0.1 to 1.5 kb. Plasmid pEH100 was digested with NdeI, dephosphorylated with alkaline phosphatase, and ligated with the genomic DNA fragments. Heat-inactivated (65°C, 10 min) ligation mixtures were used to transform *E. coli* XL1-Blue. Transformants were plated on Luria-Bertani agar with chloramphenicol. More than 2.5×10^5 colonies were recovered by flooding the agar plates with sterile PBS. Pooled suspensions containing the transformants were subjected to plasmid DNA extraction. *L. sakei* 23K was transformed with 1 to 5 μg of the plasmid DNA. Transformants were screened for in vitro active promoters (blue colonies) by plating on mMRS agar containing X-Glu and chloramphenicol. To cleanse the IVET library of constitutive and in vitro active promoters, more than 25,000 colonies exhibiting undetectable or very weak promoter activity (white or light blue colonies) were recovered by flooding the agar plates with sterile PBS. Plasmid DNA was isolated from pooled cell suspensions and used to transform *L. sakei* 23K. To determine the average insert size, DNA from 30 randomly chosen colonies of *E. coli* and *L. sakei* 23K clones was isolated and subjected to PCR with primers IVETFor and IVETgusRev.

Meat fermentation model. To simulate the ecological conditions in fermenting raw sausages, a meat fermentation model was designed. Frozen meat (beef and pork, 40% of each) and back fat (20%) were cut, minced, and stored at −20°C. Upon thawing, 6 g kg^{-1} glucose, 0.5 g kg^{-1} sodium ascorbate, 28 g kg^{-1} curing salt (sodium chloride with 0.5% sodium nitrite), and 10 mg kg^{-1} erythromycin were added to the meat under constant mixing in a KitchenAid food processor. To start the fermentation, 20 g of the meat mixture was inoculated with either *L. sakei* 23K containing the genomic library (10^8 cells), a control strain (EH100 or White [10^7 cells] or EH200 or Blue1 [10^3 cells]), or the mutant strains (10^6 cells). The mixtures were incubated microaerobically at 26°C in sterile plastic bags for 24 h. The fermented meat was propagated by back-slopping and further incubated for 24 h. A 20-g sample was then mixed with 80 ml of PBS using a

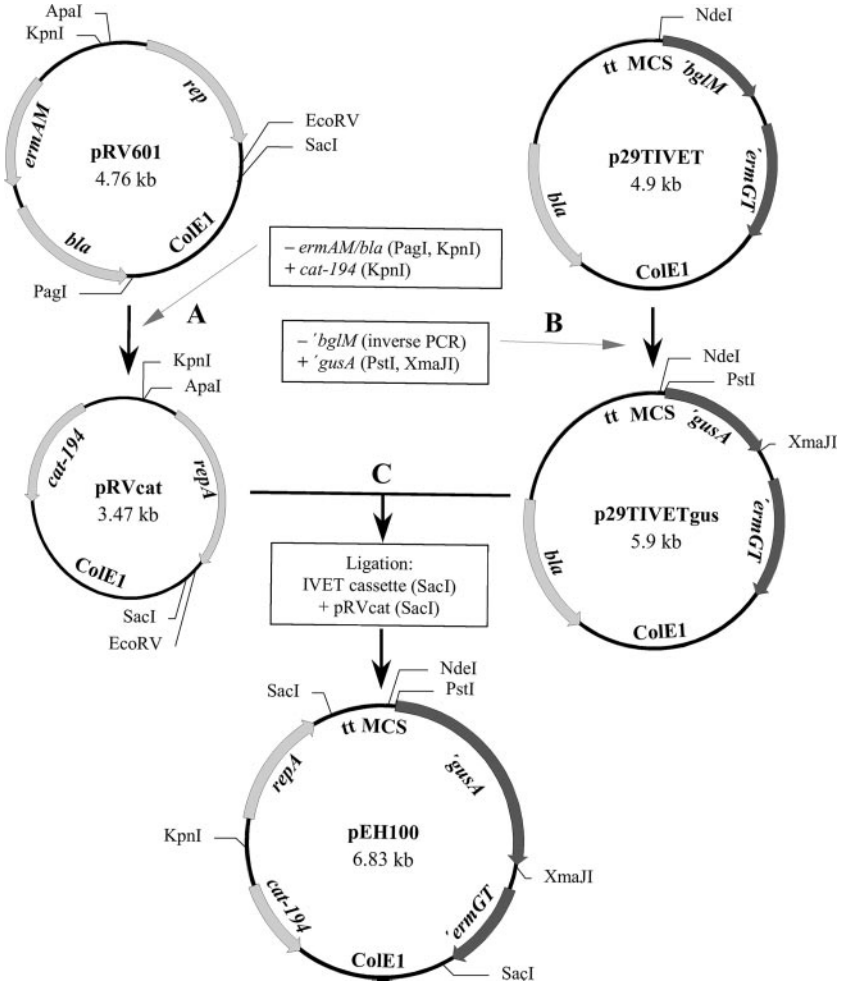

FIG. 1. Schematic illustration showing the cloning strategy for construction of the IVET vector pEH100. The IVET cassette containing promoterless reporter genes *'gusA* and *'ermGT* was designed based on the previously published IVET vector pJW100 (65). ORFs are represented as dark gray arrows (genes of the IVET cassette) or light gray arrows (other genes). Abbreviations: MCS, multiple cloning site; tt, transcriptional terminator *rrn*T1T2 from *E. coli*; ColE1, *E. coli* replication region; *repA*, *Lactobacillus* replication gene; *'bglM*, promoterless β-glucanase gene.

stomacher. A 1-ml aliquot of the suspension was used to inoculate a fresh-meat mixture (20 g).

Recovery of ici clones and identification of promoter sequences. From the sausages fermented with the IVET library, clones were recovered by growth on mMRS agar with X-Glu and chloramphenicol. Clones with no GusA activity were subcultured and stored at −85°C. The in vitro susceptibility to erythromycin was confirmed by comparing the growth on mMRS medium, supplemented with chloramphenicol (5 μg ml^{-1}) and erythromycin (10 μg ml^{-1}), with that of the control strains EH100 and EH200. The in carne induction of promoters of putative ici clones was confirmed by sausage fermentation by inoculating 10^4 cells as described above. Plasmid DNA of the ici clones was isolated and subjected to PCR with primers IVETFor and IVETgusRev. The PCR products were purified, and nucleotide sequences were determined using primers IVETFor and IVET gusRev. DNA sequencing was executed using the dye terminator cycle sequencing Quick Start kit and CEQ 8000 genetic analysis system, both supplied by Beckman Coulter Inc. (Fullerton). Sequences were compared to the genome of *L. sakei* 23K using the BLAST algorithm with a local version of the BLASTN program (http://genome.jouy.inra.fr/soft/sakei/BLAST.html) and also to sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) (2).

Construction of *L. sakei* mutants. Isogenic mutants of ici genes of *L. sakei* 23K were constructed by using pRV300 for insertional inactivation via single-crossover integration as described previously (39). Internal sequences of the ici genes *asnA2*, *ctsR*, *clpC*, LSA1194, LSA1065, LSA1637, and LSA1649 were amplified (primers are listed in Table 2), and the PCR products were digested with the restriction enzymes PstI and SalI. The fragments were ligated to the PstI/SalI-digested vector pRV300, and the ligation mixtures were used to transform *E. coli* XL1-Blue. Plasmids were used to transform *L. sakei* 23K, and their correct integration was checked by PCR using chromosomal DNA of Em^r transformants and a primer targeted against the flanking sequence of the inactivated gene in combination with a primer targeted against the cloning vector (DH05; Table 2).

RESULTS

Development of a plasmid-based IVET system for *L. sakei* 23K and construction of the genomic library. To identify *L. sakei* genes that are specifically induced during raw-sausage fermentation, the promoter trap vector pEH100 was constructed based on the endogenous monocopy *L. sakei* plasmid pRV500 (1) and the IVET cassette developed for *L. reuteri* 100-23 (65) (Fig. 1). Genomic DNA fragments of *L. sakei* 23K were inserted upstream of two transcriptionally fused, promoterless reporter genes. The first gene, *'ermGT*, confers resistance to macrolide antibiotics, and the second, *'gusA*, encodes the β-glucuronidase of *L. gasseri* ADH (55). Expression of *'ermGT* is essential for growth under the selective pressure of erythromycin. The *'gusA* gene allows differentiation on mMRS

agar plates between clones with and without in vitro promoter activity. The functionality of both reporter genes was confirmed by insertion of the constitutive *ldhL* promoter (47) in pEH100. The resulting strain, EH200, was resistant to erythromycin (MIC, >1,000 μg ml^{-1}) and formed dark blue colonies due to a strong GusA activity. In contrast, strain EH100 was highly susceptible to erythromycin (MIC, approximately 1.0 μg ml^{-1}) and showed no visible GusA activity (white colonies). Strains EH100 (Em^s $GusA^-$) and EH200 (Em^r $GusA^+$) served as negative and positive controls, respectively.

E. coli was used as an intermediate cloning host to establish the IVET library in *L. sakei*. From the resulting clone pool, two randomly picked clones were chosen as control strains. Strain Blue1 showed strong GusA activity and high erythromycin resistance (MIC, >1,000 μg ml^{-1}), whereas strain White1 had no visible GusA activity and was erythromycin sensitive (MIC, approximately 1.0 μg ml^{-1}). As clones with constitutive promoters might outnumber the ici clones during sausage fermentation, the clone pool was cleansed of clones showing in vitro promoter activity. Determination of the plasmid insert sizes of clones revealed that the size ranges from 150 to 1,800 bp, with an average size of 400 bp. Additionally, the segregational and structural stability of plasmids pEH100 and pEH200 was determined in vitro. In the case of plasmid pEH100, approximately 88% of the cells still contained the intact plasmid after 20 generations without antibiotics. Plasmid pEH200 displayed a lower stability, as approximately 80% of the cells harbored the plasmid after 20 generations. This number of generations was sufficient for selecting the clones in the IVET experiment. After 64 generations, approximately 59% and 17% of the cells contained plasmids pEH100 and pEH200, respectively.

In vivo selection and in vitro screening of *L. sakei* up-regulated genes during sausage fermentation. A meat fermentation model which reflects the ecological conditions prevailing during raw-sausage fermentation was designed. In this model, the initial phase of fermentation was simulated by continuous back-slopping of the meat mixture every 24 h. The applicability of the model in the IVET study was evaluated by inoculating the control strain EH100, EH200, Blue1, or White1 in the meat mixture. As shown in Fig. 2, after 72 h of fermentation the negative-control strains EH100 and White1 could not be detected any longer, whereas strains EH200 and Blue1 containing in vitro active promoters grew to cell counts of $>10^{10}$ CFU g^{-1} sausage. In addition, each fermentation cycle was characterized by a drop of the pH from approximately 5.9 to 5.5 (data not shown). These results indicated that the ecological conditions were suitable for growth of the *L. sakei* clones in the meat mixture and that the erythromycin concentration was sufficient for the selection of clones containing an active promoter. Thus, a batch of meat mixture was inoculated with 50,000 *L. sakei* transformants containing the IVET library. After 48 h and 72 h of fermentation, lactobacilli were screened for putative ici clones on mMRS agar plates. Approximately 1,000 clones exhibited no or very weak GusA activity, but only 114 clones were sensitive to erythromycin. Amplification and restriction digestion of the plasmid inserts from the erythromycin-sensitive clones revealed 15 different chromosomal DNA fragments, which occurred at various frequencies. To confirm the in vivo promoter activity of the inserts, the clones were subjected to meat fermentation for 24 h in the presence of erythromycin. The clones differed in their abilities to grow in the meat mixture, reaching cell counts ranging from $>10^6$ to 10^8 CFU g^{-1} sausage (data not shown).

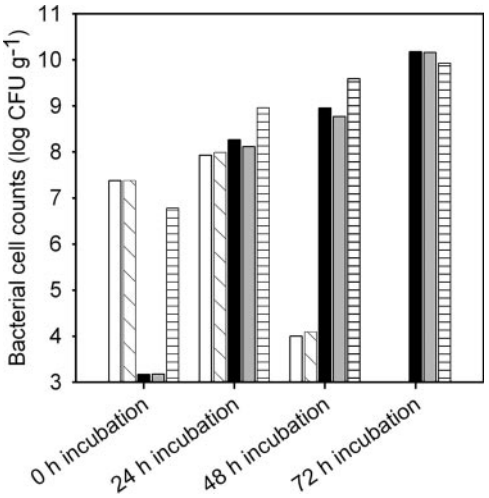

FIG. 2. *Lactobacillus* populations during raw-sausage fermentation in the presence of erythromycin. Batches were inoculated with the negative-control strain EH100 (empty bars) or White1 (diagonally striped bars) or the positive-control strain EH200 (black bars) or Blue1 (gray bars) or the IVET library (horizontally striped bars). Bacterial cell counts were determined at the beginning of fermentation (0 h incubation), after 24 h (24 h incubation), and then 24 h after the first back-slopping (48 h incubation) and 24 h after the second back-slopping (72 h incubation) by plating on mMRS agar supplemented with chloramphenicol.

Identification of ici genes. Sequence analysis of the 15 chromosomal DNA fragments (ici fragments) allowed characterization of 15 different putative promoter sequences. Several ici fragments consisted of multiple fusions of diverse chromosomal fragments. However, in all 15 cases one unambiguous putative promoter region and its corresponding open reading frame(s) (ORF) could be identified. This was accomplished by comparing the nucleotide sequence with that of the genome of *L. sakei* 23K and by considering the promoter orientation relative to the reporter genes *'ermGT* and *'gusA* (Fig. 3). Most sequences contained a putative *Lactobacillus* promoter signal (−35 region, **TT**GAC**A**; −10 region, **TA**TAA**T** [48]) and a ribosome binding site (AG**GAGG**) (both indicated by boldface). The ORFs were sorted according to the Clusters of Orthologous Groups classification (58), and the results are listed in Table 3.

Performance of *L. sakei* 23K mutants in raw-sausage fermentation. Isogenic mutants of *L. sakei* 23K were used to identify genes that are essential for the ecological performance of the organism during raw-sausage fermentation. Six ici genes were selected for mutagenesis (Table 3) on the basis of the following criteria. Genes coding for apparently essential functions (*uppS* and *pgm*), as well as genes of insufficient length for mutagenesis by homologous recombination (LSA1714, LSA0945, LSA1717, LSA0145, and LSA0121; Fig. 3 and Table 3) were excluded. Six mutant ici genes were constructed by insertional inactivation using the nonreplicative vector pRV300 (39). In addition, a mutant of gene *clpC* (LSA1779)

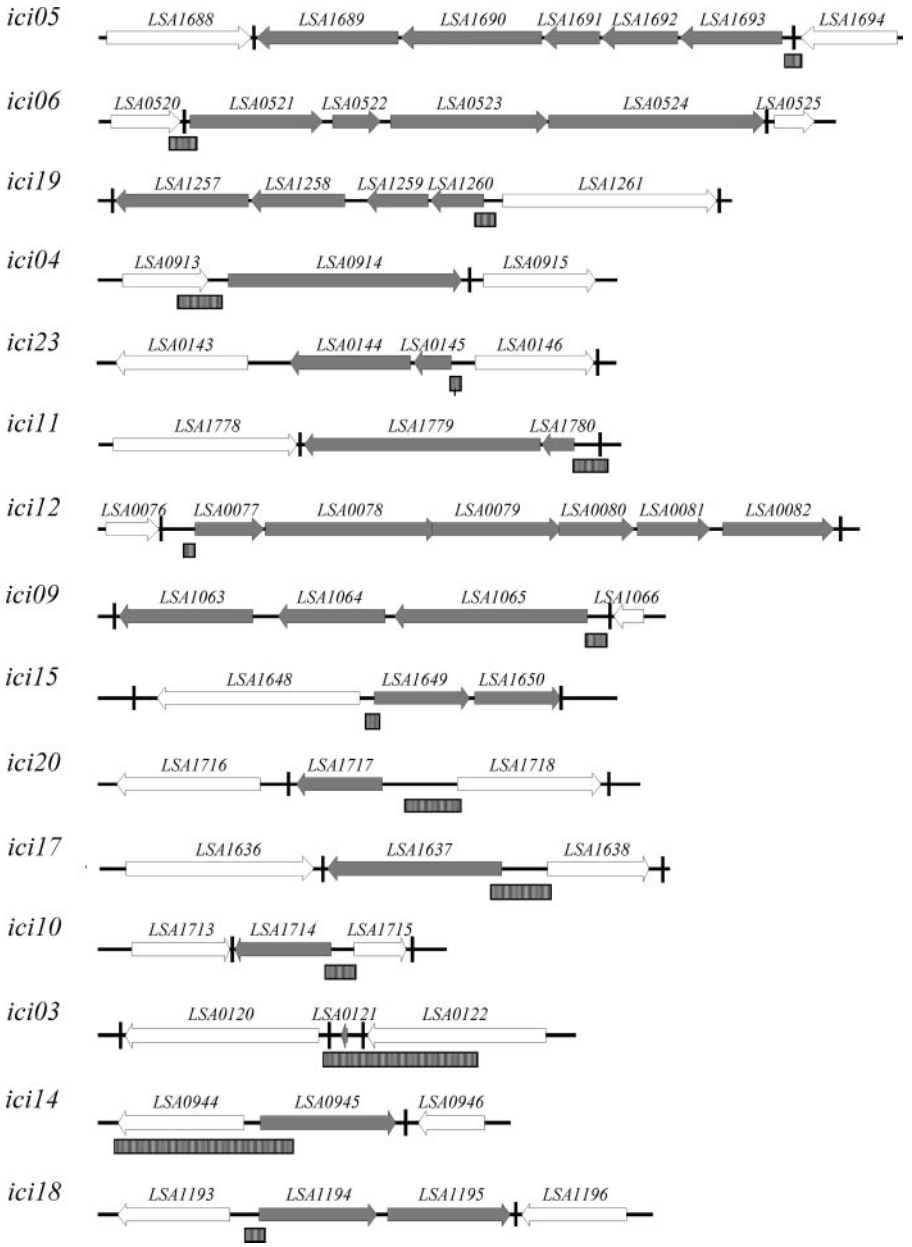

FIG. 3. Schematic representation of ici fragment localizations (dark boxes) in the genome of *L. sakei* 23K. ici genes and ORFs located downstream that might be part of a putative operon are displayed as gray arrows, adjacent ORFs as white arrows. Vertical black lines denote transcriptional terminators according to the genome annotation (GenBank accession no. NC_007576).

was constructed, as it forms a transcriptional unit together with the ici gene *ctsR* (LSA1780). Mutant RVRR3 with the ici gene *rrp-3* (Table 3) has previously been constructed (49). To investigate the effect of gene inactivation on overall performance, the growth of the mutants in mMRS medium was investigated in comparison to that of the wild-type strain *L. sakei* 23K(pLPV111), harboring plasmid pLPV111 to confer erythromycin resistance. All mutants except RVERFK grew similarly to the wild-type strain 23K(pLPV111), and only mutant RVBETA showed a slightly prolonged lag phase (Fig. 4A). Mutant RVERFK could not grow in mMRS medium, resulting in cell counts less than 10^5 CFU ml^{-1} after 30 h of incubation. It was thus excluded from further studies, as the inactivated gene appeared to be essential for effective growth.

The remaining seven mutants were tested for their ecological performance in raw-sausage fermentation and were compared to strain 23K(pLPV111). In contrast to the results obtained in mMRS medium, four mutants showed differences in growth during sausage fermentation (Fig. 4B). Compared to that of the wild-type strain, growth of mutants RVASP, RVNOD, and RVBETA was impaired to various extents. Mutant RVNOD showed the strongest growth restriction, reaching cell counts of only 10^7 CFU g^{-1} after 47 h of fermentation. In addition, a prolonged lag phase (mutant RVBETA) and

TABLE 3. *L. sakei* 23K genes that were induced during raw-sausage fermentation

Classification by Clusters of Orthologous Groups	Clone	Redundancy	Product or function (gene[s])	GenBank accession no. of gene product	Mutant strain	Genomic localization of ici fragment[a]
Amino acid transport and metabolism	ici05	1	L-Asparaginase 2 (*asnA2*, LSA1693)	YP_396306	RVASP	1675301-1675454
Carbohydrate transport and metabolism	ici06	60	Phosphoglucomutase (*pgm*, LSA0521)	YP_395133		536970-537333
Lipid metabolism	ici19	23	Undecaprenyl pyrophosphate synthetase (*uppS*, LSA1260)	YP_395871		1242610-1242876
Translation, ribosomal structure, and biogenesis	ici04	1	Asparaginyl-tRNA synthase (*asnS*, LSA0914)	YP_395525		904640-904886
DNA replication, recombination, and repair	ici23	1	Transposase of IS*1520 orfA* (*tnpA1*-IS*1520*, LSA0145)	YP_394754		140625-140698
Posttranslational modifications, protein turnover, and chaperones	ici11	2	Regulator of class III heat shock genes (*ctsR*, LSA1780)	YP_396393	RVCTSR	1757863-1758224
Signal transduction mechanisms	ici12	1	Response regulator, two-component system (*rrp-3*, LSA0077)	YP_394688	RVRR3	72282-72393
General function predicted only	ici09	2	Hypothetical metallo-β-lactamase (LSA1065)	YP_395675	RVBETA	1061711-1061893
	ici15	3	Hypothetical cell surface protein, ErfK family (LSA1649)	YP_396262	RVERFK	1634364-1634458
	ici20	2	Hypothetical transcription regulator, Xre family (LSA1717)	YP_396330		1700921-1701165
	ici17	2	Hypothetical integral membrane protein, TerC family (LSA1637)	YP_396250	RVTERC	1622922-1623152
Function unknown	ici10	5	Hypothetical small protein (LSA1714)	YP_396327		1698813-1698907
	ici03	2	Hypothetical small peptide (LSA0121)	YP_394731		112773-113780
	ici14	5	Hypothetical protein (LSA0945)	YP_395556		932126-932667
	ici18	4	Hypothetical membrane protein, nodulin-21-like/DUF125 family (LSA1194)	YP_395806	RVNOD	1175881-1175993

[a] Localization of ici fragments on the genome of *L. sakei* 23K (GenBank accession no. NC_007576).

reduced exponential growth (mutant RVASP) were also observed. On the other hand, during the first 12 h of incubation mutant RVCTSR showed increased growth with no apparent lag phase compared to strain 23K(pLPV111). Finally, mutants RVRR3, RVCLPC, and RVTERC showed growth patterns similar to that of the wild-type strain (data not shown).

DISCUSSION

By applying the IVET to *L. sakei* we identified 15 genes and/or operons which demonstrated induced expression during raw-sausage fermentation (Table 3; Fig. 3). For some of these, a role in the adaptation of the organism to the fermenting-raw-sausage environment could be deduced. The initial phase

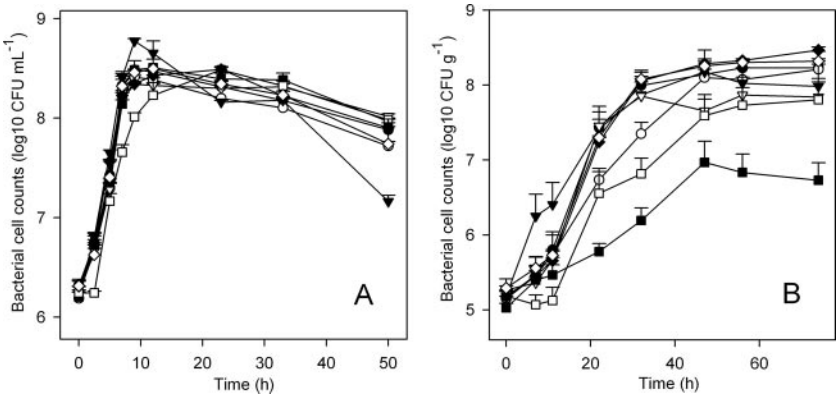

FIG. 4. Growth of *L. sakei* strains 23K(pLPV111) (●), RVASP (○), RVCTSR (▼), RVCLPC (▽), RVNOD (■), RVBETA (□), RVTERC (◆), and RVRR3 (◇) in mMRS medium (A) and during raw-sausage fermentation (B). Bacterial cell counts were determined by plating on mMRS agar supplemented with erythromycin. Values are means of two independent experiments.

of fermentation, as simulated in our raw-sausage model, is characterized by rapid growth of lactobacilli, associated with production of organic acids (most notably lactic acid) and a related rapid decrease in pH. A low pH (<5.0 after the first few days of fermentation), together with a high osmolarity due to the addition of curing salt, is responsible for the inhibition of undesirable microorganisms (37, 38). Such harsh ecological conditions may also influence the growth of the starter organism *L. sakei*. This view is consistent with our finding of several up-regulated ici genes that might be involved in the stress response of *L. sakei* to the ecological conditions prevailing in the initial phase of fermentation.

Gene *ctsR* (clone ici11) is the first gene in an operon in which *clpC* is also located. CtsR is a highly conserved transcriptional regulator of class three heat shock genes in gram-positive bacteria (19). It negatively regulates the expression of Clp proteins, some of which function as chaperones while others exhibit ATPase activity (e.g., ClpC) and form the so-called Clp proteolytic complex together with a structurally unrelated ClpP peptidase (24, 66). This complex specifically degrades misfolded proteins, an event which is crucial for the survival of bacteria under stress conditions (24, 34, 60). Although CtsR acts as a repressor of Clp expression, induction of CtsR in the course of stress response leads to subsequent degradation of the repressor itself, resulting in increased levels of Clp proteins (35). Therefore, elevated expression of *ctsR* and presumably also of *clpC*, located downstream, could be part of the stress response of *L. sakei* to the high osmolarity in the meat mixture. This assumption is consistent with the enhanced growth of the *ctsR* mutant in the raw-sausage model observed in this study (Fig. 4B). In addition, the involvement of class 3 heat shock genes in the osmotic stress response has already been demonstrated in other gram-positive bacteria, including LAB (60). For example, *Bacillus subtilis* (51) showed induction of *ctsR* and *Lactococcus lactis* (30) displayed increased production of class 3 heat shock proteins when exposed to high sodium chloride concentrations. Moreover, it was recently shown that *clpC* is induced during the passage of *Lactobacillus plantarum* through the murine gastrointestinal tract (10). As the murine gut has been identified as a stressful environment for lactobacilli (9, 65), it is tempting to speculate that the class 3 heat shock genes of lactobacilli are also involved in the general stress response.

Clone ici06 contained the promoter of the ici gene *pgm*, coding for a phosphoglucomutase. Pgm catalyzes the interconversion of glucose-6-phosphate and α-glucose-1-phosphate and represents the branching point between the glycolytic and the Leloir pathway. It has been shown that in *L. sakei* high Pgm activity is associated with high exopolysaccharide (EPS) production (17). In addition, *pgm* was shown to be induced under stress conditions, e.g., in *Streptococcus mutans* when growing under acidic conditions (68), as well as in *Lactococcus lactis* after cold shock (69). This indicates that *L. sakei* might respond to the harsh conditions during sausage fermentation by EPS production, a response that was already observed for several LAB confronted with adverse environmental conditions (31, 42, 44). However, unlike other *L. sakei* strains, 23K does not produce EPS, although some genetic information involved in EPS production, for example, cluster LSA1510 to LSA1513 (13), is present in the genome.

The *uppS* gene (clone ici19) encodes an undecaprenyl pyrophosphate synthase (UppS), a central enzyme of bacterial cell wall synthesis, and gene LSA1645 (clone ici15) may also be involved in this activity. UppS catalyzes the consecutive condensation of farnesyl pyrophosphate with eight isopentenyl pyrophosphates to form undecaprenyl pyrophosphate (C_{55} UPP), a lipid carrier for peptidoglycan precursors and also for activated nucleotide sugars used for EPS production (61, 67). LSA1645 codes for a hypothetical protein containing a conserved domain belonging to the ErfK-YbiS-YhnG protein family (pfam accession number PF03734). This domain has been shown to be essential for a peptidoglycan cross-linking enzyme of *Enterococcus faecium* which is involved in an alternate transpeptidation pathway of cell wall synthesis (7, 46). Induced expression of *uppS* and LSA1645 suggests that modifications in the cell wall and/or membrane composition are important for *L. sakei* to adapt to the adverse environmental conditions in raw sausages. This hypothesis is supported by the observation that lactobacilli respond to environmental stresses with alterations of the cell wall or cytoplasmatic membrane, e.g., changes of the ratio of saturated/unsaturated fatty acids or incorporation of certain glycolipids into the membrane (3, 22, 45, 52, 59). Interestingly, the *uppS* gene was also specifically induced during sourdough fermentation of *L. reuteri* (16), indicating an important function of this gene for the growth of lactobacilli in fermenting foodstuffs.

Sequence analysis of gene *asnA2* (ici05), coding for L-asparaginase, revealed that the protein had a moderate homology (38% amino acid identity) to *N*4-(β-*N*-acetylglucosaminyl)-L-asparaginase (aspartylglucosaminidase [AGA]) of *Flavobacterium meningosepticum* (pfam accession number PF01112). Besides generating ammonium from asparagine due to its general asparaginase activity, AGA plays a pivotal role in the degradation of N-glycans/N-linked glycoproteins by cleaving the Asn-GlcNAc linkage that joins the sugar moiety to the protein (43). Elevated expression of *asnA2* during raw-sausage fermentation could be explained in two ways. Firstly, *L. sakei* may require ammonium, because the meat substrate is in general a poor source of free nitrogen (50). However, strain 23K harbors another L-asparaginase (AsnA1) belonging to the asparaginase/amidohydrolase family (pfam accession number PF00710), which may contribute to the acquisition of ammonium. Secondly, *L. sakei* may improve its performance during fermentation by metabolizing the sugar moiety of glycoproteins occurring in the raw meat (62). Inactivation of gene *asnA2* resulted in reduced growth of mutant strain RVASP in the meat mixture. Thus, it is tempting to speculate that the degradation of N-glycans contributes to the ecological performance of *L. sakei* in raw-sausage fermentation. However, this would require an extracellular activity, but no signal peptide could be identified by in silico analysis, indicating an intracellular localization of the enzyme. Additionally, both the wild type and mutant RVASP are unable to grow on glycoproteins like ovalbumin, apo-transferrin, and fibrinogen as the sole carbon source (data not shown). Thus, it is more likely that AsnA2 plays a role in nitrogen acquisition rather than N-glycan degradation.

In bacteria, environmental signals are sensed and linked with cellular processes via two-component systems (TCS), which consist of a protein histidine kinase as the sensing unit

and a response regulator as the transcription-regulatory element (for reviews see references 8 and 12). Gene *rrp-3* (clone ici12) codes for the response regulator Rrp-3 (49) and is the first of five genes occurring in an operon. It is followed by the gene coding for the cognate protein histidine kinase Hpk-3 and three genes encoding hypothetical proteins. The Rrp-3/Hpk-3 system shows high homology to an essential TCS family (VicRK or CovRS), whose members have been shown to be important for bacteria to react to environmental stimuli, e.g., high osmolarity, extracellular Mg^{2+} concentrations for streptococci (25, 41), and cold shock induction in *L. lactis* (69). Rrp-3/Hpk-3 might therefore possess a similar function in *L. sakei*. However, the *rrp-3* mutant was not impaired in its performance during sausage fermentation. This is consistent with the previous finding that the Rrp-3/Hpk-3 system of *L. sakei* is not involved in responses to acid, temperature, and oxidative stress (49), as well as to high osmolarity in mMRS medium (data not shown).

A sequence homology search revealed that the product of gene LSA1065 (clone ici09) possesses the β-CASP functional domain, a characteristic domain for a new family of RNA-metabolizing metallo-β-lactamases (pfam accession number PF07521) (11). In *B. subtilis*, the members RnjA (YkqC) and RnjB (YmfA) of this enzyme family act as endoribonucleases that appear to be implicated in regulatory processing and maturation of specific mRNAs and may therefore be important for a fast adaptation of the organism to nutritional and environmental changes (21). LSA1065 could be an ortholog of this family in *L. sakei*, playing a role in posttranscriptional regulation. As mutant RVBETA exhibited a prolonged lag phase during sausage fermentation, it is tempting to speculate that expression of LSA1065 is part of the adaptive response of *L. sakei* to the ecological conditions in meat fermentation.

The hypothetical product of gene LSA1194 (clone ici18) showed weak homology to members of the nodulin-21-like/DUF125 family of integral membrane proteins (pfam accession number PF01988). Representatives are nodulin-21, a plant nodule-specific protein that may be involved in symbiotic nitrogen fixation (18), and CCC1, a yeast vacuole transmembrane protein that plays a role in Ca^{2+} homeostasis and functions as an iron and manganese transporter (23, 36). Orthologs are widespread among pro- and eukaryotic organisms, with frequent occurrence of multiple copies per genome, pointing to a conserved function for these membrane-associated proteins. *L. sakei* 23K harbors two additional ORFs that encode the nodulin-21-like domain (LSA1524 and LSA1195). Interestingly, the inactivation of LSA1194 leads to a reduced performance of *L. sakei* during raw-sausage fermentation (Fig. 4B); however, no conclusions can be drawn about the function of this hypothetical protein.

The use of IVET facilitated a first insight into the transcriptional response of *L. sakei* during raw-sausage fermentation. The results of this study are of importance in several ways. Firstly, they contribute to the knowledge of properties required for the ecological adaptation of *L. sakei* to the meat environment. Secondly, the information can be used for the selection and development of improved starter organisms. As the sausage model used in this study has been designed to simulate the initial fermentation phase, the results may also give an indication as to which genes need to be expressed in order to maximize the organism's competitiveness. This information may be of importance for production of starter cultures, for example, by preconditioning the cells at the end of growth in the fermentor to obtain expression of genes that are required in the initial phase of meat fermentation.

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